

## INTRODUCTION

Children differ from adults in many pharmacotherapeutic aspects [1] which have driven the design of paediatric drugs. In this respect, features, such as, ease and safety of administration, dose uniformity and flexibility, palatability, safety of excipients, stability and therapeutic equivalency of paediatric dosage forms [2]. While encapsulation has shown great potential regarding the development of innovative and improved drug products, milk is considered as a promising platform for paediatric drug delivery [3].

In the present work, milk protein micelles incorporating model drugs, relevant in child treatment (paracetamol and vitamin A), have been produced and evaluated as drug delivery vehicles. The ability of chemical crosslinking of milk micelles to improve its thermodynamic stability and tailor drug release was also assessed.

## METHODOLOGY

### PRODUCTION OF CASEIN MICELLES

Fresh skimmed milk was purchased from Mimosa (Portugal) and all reagents were of analytical grade (> 99% purity). Milk proteins (mainly casein; see results) were extracted from fresh milk, using a combination of pH (4.1-4.3) and heat treatment (55°C), vacuum filtered and dried (40°C), until constant weight. Proteins extracted were identified and quantified by Coomassie blue stained SDS-PAGE densitometry. Protein (75mg/ml) dispersions in PBS (pH 7.4), containing 0.5% (w/v) lecithin and 4% (v/v) Tween 80, were produced under magnetic stirring (100 rpm). Drugs (paracetamol or vitamin A) were then added in increasing amounts and micelle formation promoted by pH drop (4.1-4.3) with lactic acid; drug-loaded micelles were filtered, washed and freeze-dried. Crosslinking was carried out, using 0.1M of EDAC in a water-acetone (2:8) solution (24h, 5°C, 300 rpm) and the crosslinking degree estimated from the remaining free amino groups by the ninhydrin method. Crosslinked (CL) micelles were air-dried until constant weight.

### CHARACTERIZATION OF CASEIN MICELLES

Micelles were characterized for particle size, zeta potential and FTIR spectra (4000-400 cm<sup>-1</sup>, resolution 4cm<sup>-1</sup>, 30 scans). Drugs were quantified by UV/vis spectrometry (325nm - vitamin A; 243nm - paracetamol) and the encapsulation efficiency (EE;% ) determined. The release of paracetamol from uncrosslinked (UCL) and CL micelles was assessed from samples taken at time intervals, upon dialysis (PBS, pH 7.4, 37°C, 100 rpm) of paracetamol from a 10 kDa cut-off membrane. Release studies in the presence of proteolytic enzymes were also conducted using trypsin (0.3%; w/v). All measurements were made in triplicate.

## RESULTS AND DISCUSSION

### SDS-PAGE DENSITOMETRY

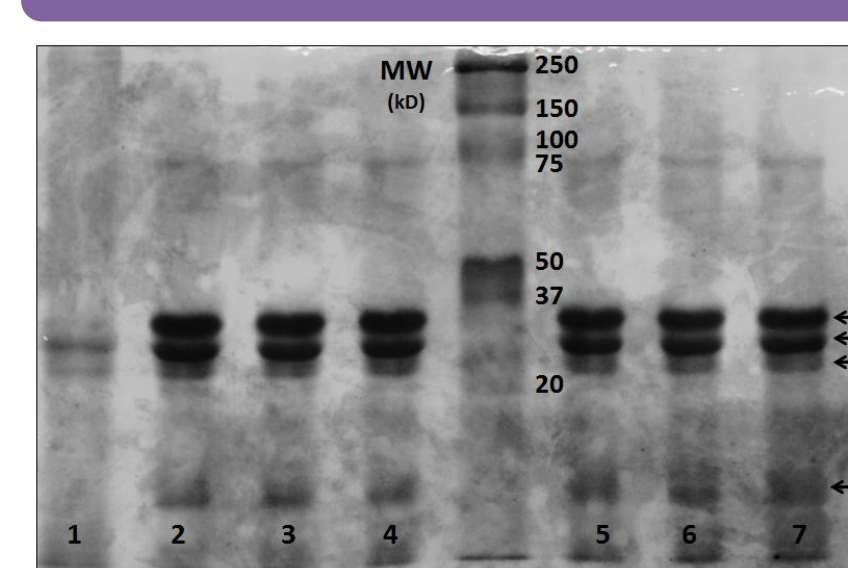


Fig. 1 – Coomassie blue SDS-PAGE of skimmed milk protein extracts

1) Control; 2) F0; 3) F1; 4) F2; 5) F3; 6) F4; 7) F5

Table 1 – Percentage of casein (CN) and whey proteins (β-Lactoglobulin) assessed by densitometry of SDS-PAGE bands

Samples	CN:drug ratio	Vitamin A		Paracetamol	
		Casein (%)	β-LG (%)	Casein (%)	β-LG (%)
F0	0:3	80.82	19.18	87.48	12.52
F1	0.09:3	89.94	10.06	87.65	12.35
F2	0.18:3	89.16	10.84	85.72	14.28
F3	0.51:3	86.48	13.52	84.63	15.37
F4	0.63:3	82.76	17.24	90.93	9.07
F5	1:3	90.14	9.86	88.72	11.28

- Casein represents 81-91% of the proteins' total mass and β-lactoglobulin 9-19%
- More soluble, as compared to commercial casein used in preliminary experiments

### PARTICLE SIZE AND ZETA POTENTIAL

Table 2 – Particle size and Zeta Potential of different micelle formulations

	Vitamin A					Paracetamol				
	F0	F1	F2	F3	F4	F0	F1	F2	F3	F4
Particle Size (nm)										
UCL	175.30 ± 25.27	186.80 ± 34.81	208.40 ± 59.25	162.30 ± 22.70	161.40 ± 23.72	167.40 ± 18.79	180.60 ± 3.79	143.30 ± 9.71	170.40 ± 14.73	177.30 ± 25.36
CL	249.60 ± 39.88	380.50 ± 155.00	266.1 ± 142.40	317.20 ± 46.54	292.60 ± 114.90	300.50 ± 50.83	290.80 ± 53.44	285.10 ± 30.45	328.20 ± 152.20	255.10 ± 161.50
Zeta Potential (mV)	-22.0 ± 1.65	-20.9 ± 2.56	-19.3 ± 1.53	-24.1 ± 3.37	-27.1 ± 2.02	-20.3 ± 1.04	-20.1 ± 5.05	-22.1 ± 0.52	-21.40 ± 1.19	-15.4 ± 1.29

- CL promoted formation of larger micelles (≈300nm) vs UCL samples (≈170nm)
- Increased size distribution, consistent with occurrence of intermolecular CL
- Negative surface charge (-15 to -22 mV), with zeta potential magnitude, indicative of redispersibility

### MICELLES PREPARATION AND DRUG ENTRAPMENT

Table 3 – Yield, Crosslinking Degree and Encapsulation Efficiency of skimmed milk protein micelles

Samples	CN:drug ratios	Vitamin A			Paracetamol		
		Yield (%)	CL degree (%)	EE (%)	Yield (%)	CL degree (%)	EE (%)
F0	0:3	82.91	70.04	0.00	87.07	83.94	0.00
F1	0.09:3	84.25	49.45	1.55	84.93	85.65	3.65
F2	0.18:3	89.16	53.47	1.56	90.00	79.60	5.45
F3	0.51:3	94.02	79.72	1.94	91.73	81.84	12.07
F4	0.63:3	89.78	72.64	1.98	87.33	87.80	15.90
F5	1:3	93.15	65.25	1.96	87.07	77.83	31.39

- Yield: 83-94% for vitamin A and 85-92% for paracetamol; Crosslinking degree: 54-80% and 78-88%

- Micelles unable to entrap vitamin A (EE up to 2%)
- Paracetamol: EE increased with amount of drug (up to 31%, for 1:3 protein:drug ratio)

## CONCLUSION

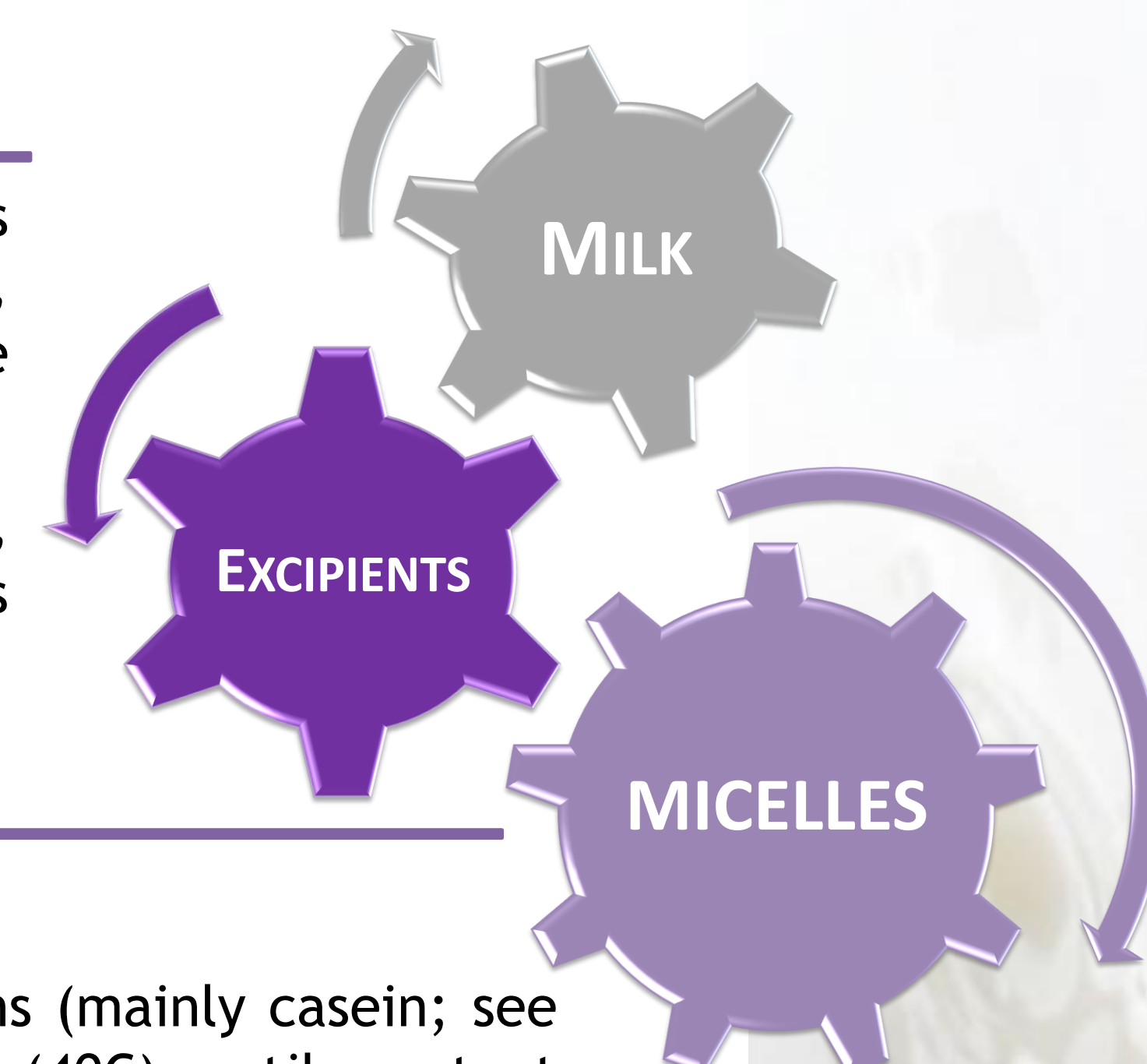
Micelles produced from fresh milk proteins are promising prolonged-release carriers for drug delivery in paediatrics, which are also expected to be safe, provided the crosslinking reaction is controlled to minimize intra- and intermolecular crosslinking. Experiments are under way to ascertain if the use of fat milk would promote encapsulation of hydrophobic drugs.

## REFERENCES

- Bartelink, I.H., Rademaker, C.M.A., Schobben, A.F.A.M., Van Den Anker, J.N. (2006). Clin. Pharmacokinet. 45, 1077-1097.
- Ali, A.A., Charoo, N.A., Abdallah, D.B. (2014) Drug Dev. Ind. Pharm. 40, 1283-1299.
- Aguiar J.P., Fernandes T.A.P., Nese C., Fernandes A.I., Pinto J.F. (2017) J Pharm Pharmacol. 69, 554-566.

## ACKNOWLEDGEMENT

Fundação para a Ciência e a Tecnologia (PTDC/DTP-FTO/1057/2012) is gratefully acknowledged for funding.



### CROSSLINKING DEGREE (FOLLOW-UP)

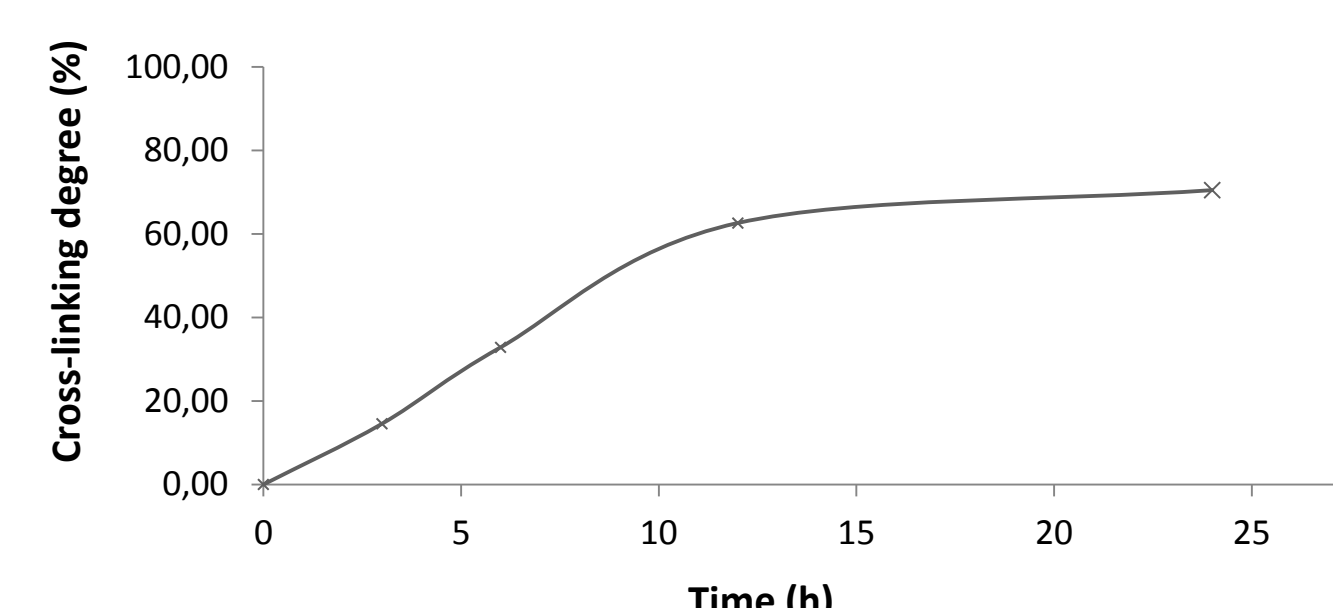


Fig. 2 – Crosslinking degree of skimmed milk protein micelles

CL estimated from the remaining free amino groups by the ninhydrin method

- CL steadied after 10h, but the reaction was left to proceed during 24h for maximum crosslinking

### FT-IR SPECTRA ANALYSIS

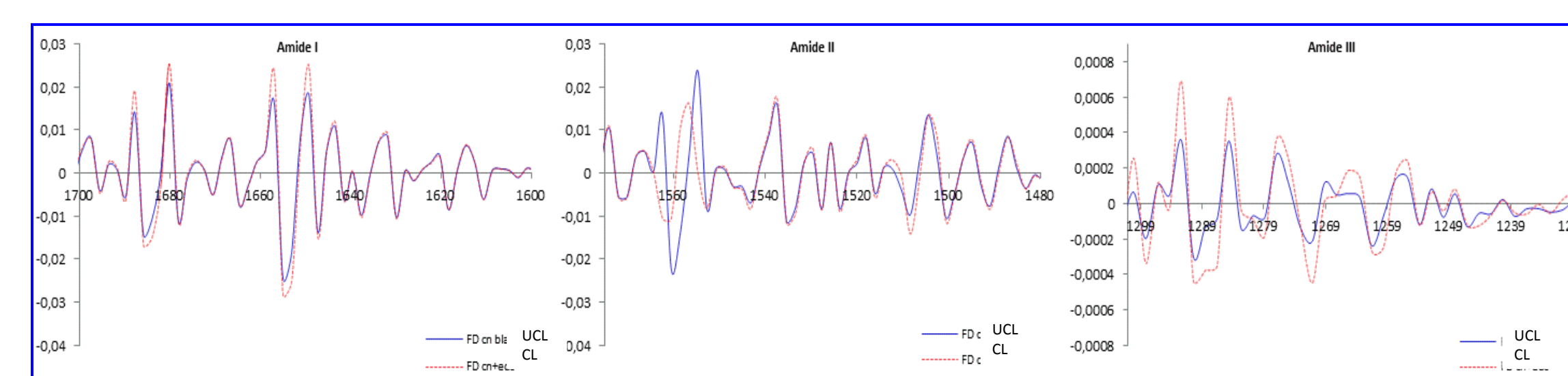


Fig. 3 – Second derivative amide I, II and III spectra of crosslinked (CL) and uncrosslinked (UCL) micelles

- FT-IR bands of paracetamol in micelles predominantly invisible due to interference of casein bands
- Second derivative spectra of CL micelles with altered stretching frequencies and peak shifts in amides regions
- Differences might be attributed to potential intramolecular crosslinking

### DRUG RELEASE STUDY

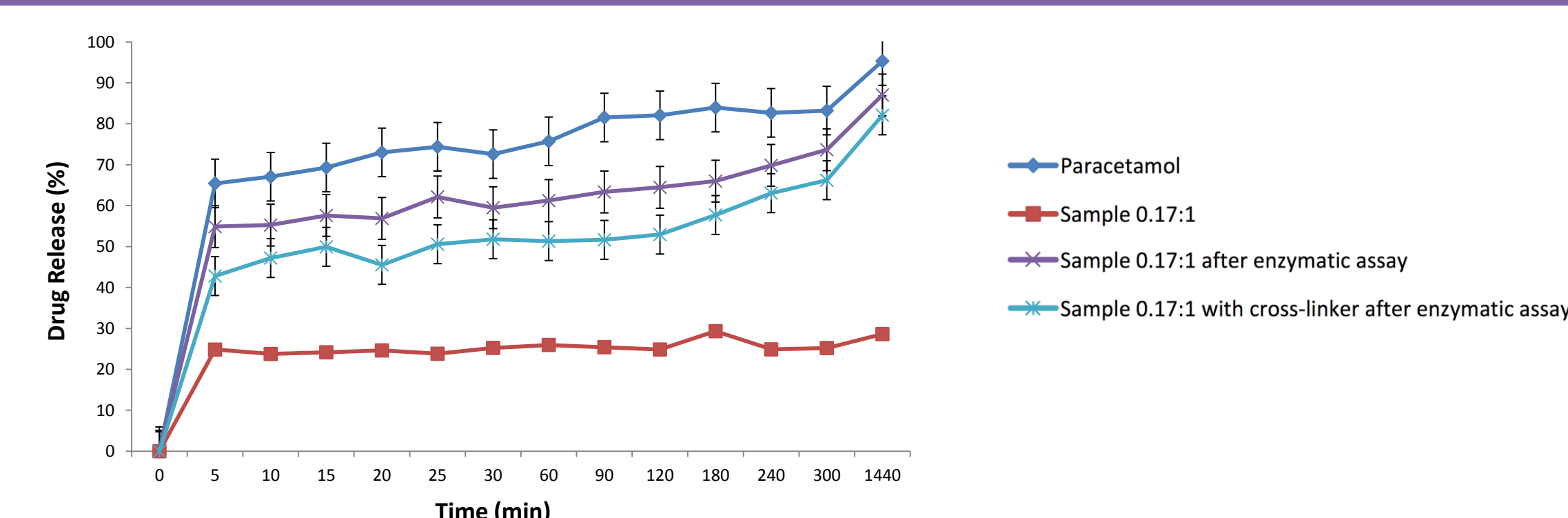


Fig. 4 – Paracetamol release (%) from protein micelles

- Release of paracetamol was retarded upon encapsulation, particularly in CL micelles